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Obesity, non-alcoholic fatty liver disease, and atherothrombosis: a role for the intestinal microbiota?

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Abstract

Whereas the association between intestinal microorganisms and health has been widely accepted in the area of infectious disease, recent advances have now implied a role for the intestinal microbiota in human energy balance. In fact, numerous studies support an intricate relationship between the intestinal microbiota and obesity, as well as subsequent insulin resistance and non-alcoholic fatty liver disease. Intestinal microorganisms also seem to be involved in haemostatic tone and atherogenesis. However, as most of the findings stem from observational data, intervention studies in humans using interventions selectively aimed at altering the composition and activity of the intestinal microbiota are crucial to prove causality. If substantiated, this could open the arena for modulation of the intestinal microbiota as a future target in obesity-associated disease, both as a diagnostic test for personalized algorithms and for selective therapeutic strategies.

Keywords: Atherosclerosis, atherothrombosis, intestinal microbiota, non-alcoholic fatty liver disease, obesity, thrombosis
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Introduction

Obesity is an increasingly important public health issue, as it is expected that, by 2030, one-third of the population of the western world will be obese [1]. The term 'metabolic syndrome' is widely used as a clinical definition of overweight individuals at increased risk of a large series of comorbidities, such as insulin resistance, cardiovascular disease (CVD), venous thromboembolic events, and non-alcoholic fatty liver disease (NAFLD) [2] (Fig. 1) . Apart from many other genetic risk factors that play a role in the development of obesity [3], metabolic syndrome is increasingly thought to be a chronic inflammatory disease driven by chronic intestinal bacterial translocation resulting in endotoxaemia [4,5]. Endotoxaemia is characterized by Gram-negative bacterial capsule fragments in the plasma, and is linearly associated with the concentration of lipopolysaccharide-binding protein in plasma. In this regard, plasma lipopolysaccharide-binding protein was found to be a marker of chronic inflammation associated with the development of obesity and insulin resistance in both mice and humans [6,7]. Moreover, there is increasing evidence that the intestinal microbiota might contribute to host metabolism and obesity [8], a process that is thought to stem from impaired gut barrier function in obese subjects [9].

The average human bowel is home to trillions of microorganisms, mainly bacteria, but also viruses and a low number of fungi, which outnumber the cells of their human host by a factor of ten to one [10]. In fact, their genes even outnumber the human genes by >100-fold. Microbial colonization starts soon after birth and, although the initial composition of the microbiota varies, it becomes relatively stable after the age of 3–4 years, and remains so into advanced age [10]. The intestinal microbiota serves as an 'exteriorized' organ, complementing and interacting with human metabolism, and so giving rise to novel therapeutic targets. In this respect, the composition of the intestinal microbiota and its collective

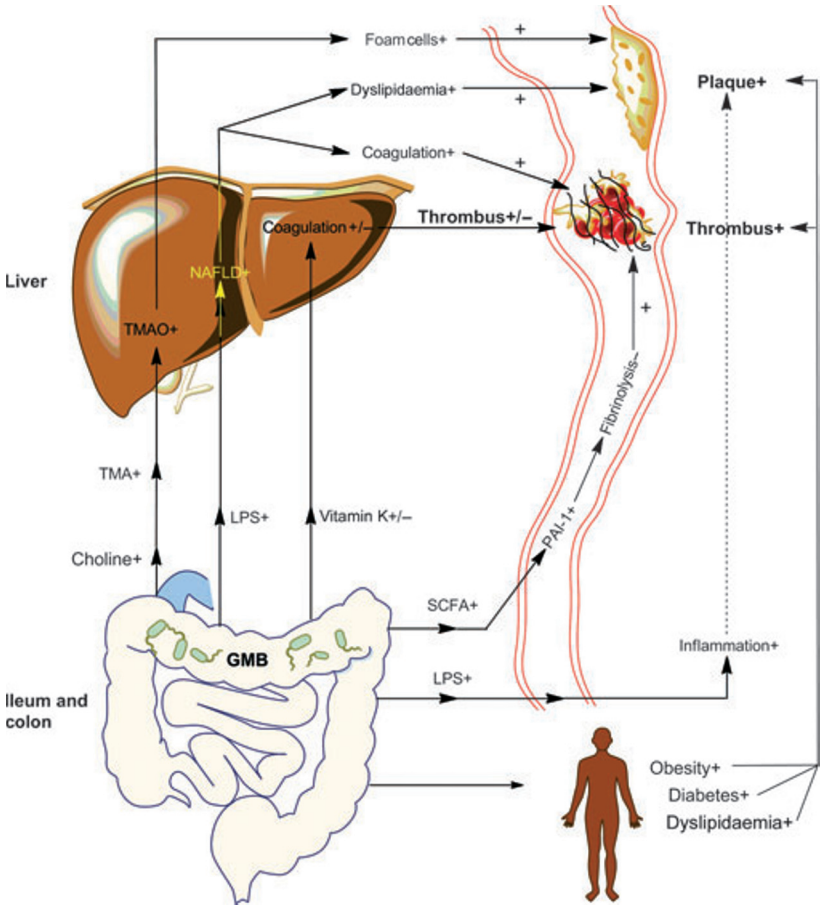

FIG. 1. Potential associations between the intestinal microbiota and obesity-related comorbidities, comprising insulin resistance, cardiovascular disease, venous thromboembolic events and non-alcoholic fatty liver disease (NAFLD). GMB, Gut microbiota; LPS, lipopolysaccharide; PAI-1, plasminogen activator inhibitor-1; SCFA, short-chain fatty acid; TMAO, trimethylamine-*N*-oxide.

genome (also known as the microbiome) is considered to be an important factor in various diseases, ranging from gastrointestinal tract disease to obesity [11]. Bacterial numbers and composition vary considerably along the human gastrointestinal tract. In the oral cavity, there are approximately 10^{12} bacteria; the numbers in the stomach and small intestine are significantly lower, owing to the rapid transit times and the secretion of gastric acid, and bile and pancreatic juice. Bacterial numbers range from 10^{0} to 10^{4} per gram in the stomach, whereas the proximal small intestine and the ileum harbour 10^{5}–10^{7} and up to 10^{7}–10^{8} bacteria per gram of intestinal content, respectively. The highest number of bacterial cells is found in the large intestine, with approximately 10^{11} per gram of intestinal content [12]. Quantitatively, the most important phyla of the intestinal microbiota include the *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Proteobacteria*, and *Verrucomicrobia*, the representatives of which are often regarded as strict anaerobes (except for the *Proteobacteria*, which can be facultative anaerobes) [12]. Older literature has suggested that germ-free animals are less susceptible to obesity [13] and atherosclerotic plaque [14] and thrombus formation [15], suggesting that the intestinal microbiota may play a role in these cardiometabolic diseases [16]. Until recently, knowledge of the intestinal microbiota was limited, mainly because of the lack of methods for growing and identifying their representatives. The introduction of culture-independent approaches based on analysis of 16S rRNA and its corresponding genes has profoundly changed the landscape; these topics have already been covered in recent reviews [11,17,18]. Nevertheless, owing to the use of different analysis platforms, reproducibility issues, and other biases, including concomitant medication use in selected subjects, inconsistent results have been generated by these observational studies, and this calls for caution in their interpretation [17]. In the present article, we will discuss the potential influence of the intestinal microbiota on the development of obesity-associated parameters such as NAFLD and hypercoagulability. The underlying pathophysiological processes, including microbiota-associated chronic low-grade inflammation, and potential therapeutic targets are also described.

Gut microbiota, inflammation and lipid metabolism

It is widely acknowledged that obesity and subsequent insulin resistance are closely related to the presence of adipose tissue inflammation. As adipose tissue is important for the production of various inflammation cytokines [19], there is ample

evidence that visceral adipose tissue and, to a lesser extent, subcutaneous adipose tissue drive the development of insulin resistance [20]. More recently, the numbers of visceral adipose tissue macrophages and crown-like structures (or accumulated CD68-positive macrophages) were found to correlate with inflammatory gene expression [21], suggesting a role for the innate immune system in this process [22]. Likewise, changes in the intestinal microbiota could induce a chronic inflammatory tone in obesity [23], which drives activation of pattern recognition receptors, such as toll-like receptors (TLRs), by bacterial endotoxins (e.g. lipopolysaccharide (LPS)) that bind to TLRs or other pattern recognition receptors, thus activating the innate immune system [24]. It should, however, be noted that, in addition to LPS, the intestinal microbiota is a source of many other proinflammatory molecules, such as peptidoglycans [25] and flagellins [26], which are also able to activate inflammatory pathways [27]. Nevertheless, several lines of evidence suggest a causal relationship between endotoxaemia and obesity, as LPS infusion in mice [24] resulted in fasting hyperglycaemia/dyslipidaemia and hyperinsulinaemia, hepatic insulin resistance, obesity, hepatic steatosis, and macrophage infiltration of visceral adipose tissue. Interestingly, these effects were also found when mice were put on a high-fat diet for 4 weeks. To further demonstrate the relationship between LPS and these effects, CD14-knockout mice (CD14 forms a complex with TLR4 that is vital to the innate immune system for binding of LPS) were given the same LPS infusion, and these mice proved to be resistant to the development of low-grade inflammation and dyslipidaemia. With hindsight, this may not have come as a surprise, as previous studies suggested an important role for plasma lipoproteins in the clearance of endotoxins, including very low-density lipoprotein particles [28] and high-density lipoprotein cholesterol [29,30], whereas postprandial chylomicronaemia was associated with an increased plasma endotoxin load [31]. On the other hand, the intestinal microbiota composition itself can also affect lipid metabolism [27], as bacterially produced short-chain fatty acids (SCFAs) affect lipolysis [13,32]. Together, these observations suggest bi-directional cross-talk between the intestinal microbiota, chronic inflammatory tone, and SCFA metabolism.

Gut microbiota, obesity, and NAFLD

NAFLD is closely related to obesity, metabolic syndrome, and insulin resistance [33], and its worldwide prevalence is rapidly increasing [34]. The most extreme form of NAFLD is non-alcoholic steatohepatitis (NASH), a chronic liver disease that has the potential to progress to liver cirrhosis, liver failure, and hepatocellular carcinoma [35]. An increasing amount of data showing a causal link between the small-intestine microbiota and NAFLD in animals has emerged [4,36], all hinting at separate pathophysiological pathways. First, the postprandial increase in the plasma endotoxin concentration in the portal vein may exceed the clearance capacity of the hepatic Kupffer cells in individuals with obesity [31], resulting in systemic endotoxaemia and low-grade chronic inflammation [37]. Indeed, recent data indicate that exogenous stimuli, such as dietary choline, can directly drive the intestinal microbial composition and subsequent intestinal permeability and NAFLD/NASH [36,38,39] . This metabolic state is mostly mediated by bacterial endotoxins such as LPS [40] derived from intestinal bacteria (e.g. proteobacteria) that are found in relatively large numbers in the intestines of obese subjects [9]. As previously mentioned, obese individuals are thought to have increased intestinal permeability, partly because of TLR4 induction [41] and altered tight junction proteins [42], potentially resulting in an increased endotoxin load in the portal vein, with ensuing overload of the hepatic Kupffer cells. This results in a so-called 'first hit'. In this 'first hit', liver injury occurs through cytokine release (tumour necrosis factor-α) and fibrogenesis [43]. However, constant activation of TLRs in Kuppfer cells leads to a decrease in TLR tolerance (second hit), inducing chronic liver disease [44]. Indeed, chronic exposure to low-dose LPS in mice has been shown to induce NASH, whereas TLR4 knockout mice (lacking the receptor that binds LPS and consequently activates the innate immune system) seem to be protected, suggesting a potential causal link between obesity, the intestinal microbiota, and the development of NASH [45].

Gut microbiota, obesity, and coagulation

Obesity and metabolic syndrome are intrinsically linked and are associated with NASH and hypercoagulability [2,33,46]. In this respect, increased thrombin generation dependent on vitamin K-dependent clotting factors (II, VII, IX, and X) has been demonstrated in obese humans [47]. In this respect, the liver contains the largest (90%) vitamin K pool, which is derived from two natural vitamins. These are phylloquinone (also known as vitamin K_1), which comes from the diet (in particular, vegetables), and menaquinone (also known as vitamin K_2), which is produced by intestinal bacteria [48]. It was recognized long ago that the amount of endogenous vitamin K synthesis by the microbiota (e.g. the *Firmicutes*) in the distal intestine greatly exceeds the required daily dose required to prevent haemostatic disorders [49]. Indeed, hepatic vitamin K_1 stores are depleted in healthy subjects without affecting haemostatic tone within days after dietary vitamin K restriction, as levels of intestinal microbiota-derived vitamin K_2 remain stable for several weeks [50]. Other indirect evidence for a role of the intestinal microbiota in haemostatic activity stems from intervention studies with vitamin K antagonists in healthy subjects. Administration of

vitamin K_2 alone directly increases factor VII plasma levels in these subjects, resulting in a normalized haemostatic tone and suggesting a role for bacterially synthesized vitamin K_2 in overall human haemostasis [51]. With respect to a causal role of the intestinal microbiota in hypercoagulability, direct evidence is limited. Reinhardt *et al.* [52] showed that intestinal tissue factor levels are regulated by the intestinal microbiota. More indirect data suggest a regulatory role for the intestinal microbiota in the inhibition of fibrinolysis through plasminogen activator inhibitor-1 via SCFAs produced by the gut microbiota [53]. This is of particular interest, as obese human subjects are reported to have reduced intestinal SCFA production [8]. Moreover, a strong correlation between obesity, NAFLD and hypercoagulability has been described [2,33,46,54]. For example, obesity is associated with increased plasma concentrations of procoagulant factors (tissue factor, factor VII, and fibrinogen) and decreased fibrinolysis [2] (increased plasminogen activator inhibitor-1 activity and decreased tissue plasminogen activity). In line with this, the levels of plasma coagulation factors VIII, IX, XI and XII are increased in patients with NAFLD, and are positively related to hepatic fat content [55]. This hypercoagulability directly leads to an increased risk of venous thromboembolic events [56]. Nevertheless, it is still unclear which small-intestine or large-intestine bacterial species are involved in the aetiology of this hypercoaguable state, and to what extent targeted intervention can normalize the intestinal microbiota composition in obese humans with NAFLD.

Gut microbiota, obesity, and atherosclerosis

As current therapeutic regimens prevent only 25% of all cardiovascular events, atherosclerosis still remains one of the major health problems throughout the western world, in particular in subjects with moderate to severe obesity [57]. It has been well established that obesity, NAFLD and dyslipidaemia are important risk factors for the development of atherosclerosis and subsequent CVD [58]. In addition, chronic low-grade inflammation induced by intestinal microbiota-derived endotoxaemia was recently found to be a risk factor for obesity, NAFLD, and insulin resistance [59]. In the Bruneck study, plasma endotoxin levels above the 90th percentile were associated with a three-fold increase in cardiovascular event risk [60], and animal experiments clearly demonstrated that endotoxin injection accelerates cholesterol-induced atherosclerosis [61]. In this section, we will therefore focus on the role of the intestinal microbiota in the induction of an inflammatory state ultimately contributing to accelerated atherogenesis.

As previously mentioned, another driving factor for chronic low-grade endotoxaemia might be the co-transport of intestinal bacteria via intestinal uptake of dietary lipoproteins (chylomicrons) [62]. Recent data have shown that dietary fat promotes intestinal absorption of LPS from the gut microbiota in apical intestinal epithelial cells, which may subsequently contribute to various inflammatory disorders [63]. In this respect, several studies have reported on the association between the intestinal microbiota and obesity and its sequelae, dyslipidaemia and insulin resistance [64], as germ-free mice on a high-fat diet were characterized by lower plasma cholesterol concentrations and less hepatic triglyceride, phosphatidylcholine and tricarboxylic acid accumulation than conventionally raised mice [65]. Interestingly, these hepatic lipids have also been implicated in the development of human NAFLD [66]. Dietary phosphatidylcholines, for example, were directly related to the intestinal microbiota and subsequent CVD, as these lipids are catabolized by the gut microbiota into trimethylamine, which is subsequently converted to trimethylamine-*N*-oxide (TMAO) in the liver [16]. In this landmark study, it was shown that plasma levels of choline and TMAO are directly correlated with the percentage of foam cells in atherosclerotic lesions of conventionally raised mice, as well as the degree of dyslipidaemia and cardiovascular events in humans. The causal relationship between TMAO, the intestinal microbiota and CVD was underscored by the fact that broad-spectrum oral antibiotic treatment significantly reduced TMAO plasma levels and atherosclerotic lesion formation in mice. In line with this observation, obese patients with NAFLD are characterized by dyslipidaemia, increased oxidative stress, and an increased atherosclerotic burden [67–69]. Thus, these data lend further support for a relationship between the intestinal microbiota, NAFLD and chronic inflammation driving dyslipidaemia and cardiovascular inflammation.

Gut microbiota, NAFLD and atherothrombosis in obesity: novel therapeutic options

As the number of studies on the intestinal microbiota is increasing, the number of associations with this biological factor are increasing [11]. To date, animal models have been mainly used to study the associations with the intestinal microbiota. Although they are useful in providing mechanistic insights, these results cannot be directly extrapolated to humans. Nevertheless, these findings may hint at potentially useful therapeutic interventions, which obviously should be validated in the human clinical setting.

First, the intestinal production of vitamin K_2 can be disrupted by short-term oral antibiotic use. It was shown in post-mortem liver samples that there was a five-fold reduction in the endogenous vitamin K_2 content in individuals who had received broad-spectrum antimicrobials prior to death [70]. Moreover, similar effects of antibiotics on haemostatic tone have been reported in humans [71], with an increased incidence of hypothrombinaemia being related to the eradication

of intestinal vitamin K_2-producing bacteria [72]. Although more research is needed, one could hypothesize that the administration of specific microbial strains as probiotics may affect the biosynthesis of hepatic clotting factors, and that they could serve as natural anticoagulants in obese subjects.

Second, bacterial DNA from *Porphyromonas gingivalis* has been isolated in human carotid plaque material, reflecting the presence of naturally occurring microorganisms of the oral cavity [73] and gut [74] in atheroma. Older data had already suggested a strong relationship between *Chlamydia pneumoniae* infection, acute coronary syndrome, and atherosclerosis [75]. Unfortunately, all randomized controlled intervention trials [76] aimed at reducing cardiovascular events by targeted antibiotic treatment have proven unsuccessful in patients at increased cardiovascular risk. Statins, however, are known to lower CVD risk even in apparently healthy men and women with chronically increased inflammatory tone [77]. Interestingly, statins might reduce the number of bacterial infections and endotoxaemia-induced inflammation by blocking TLR activation [78,79]. As a recent study suggested a direct effect of the intestinal microbiota on statin metabolism [80], it is tempting to speculate about potential beneficial effects of statin therapy on the small-intestine microbiota composition, reduced bacterial translocation, and subsequent CVD. Likewise, 6 months of treatment with the peroxisome proliferator-activated receptor-γ agonist pioglitazone led to metabolic and histological improvement of NASH in obese subjects [81]. As *in vitro* experiments demonstrated that peroxisome proliferator-activated receptor-γ activation can reduce the pro-inflammatory capacity of intestinal bacteria [82], it is tempting to speculate that part of the above-mentioned clinical improvement could be induced via the altered small-intestine microbiota composition induced by glitazones.

Finally, a recent study suggests that the intestinal microbiota metabolites can positively affect the reverse cholesterol transport pathway by affecting ABCA1/ABCG1-cholesterol-mediated efflux [83]. Although data on the role of intestinal cholesterol metabolism and microbiota are not available for humans, a direct correlation between actinobacteria and low-density lipoprotein cholesterol has been reported, and a set of potentially pathogenic proteobacteria showed a negative correlation with phosphatidylcholines [84]. Moreover, we have recently reported on our experience with the effect of healthy-donor gut microbiota infusions (or faecal microbiota transplantations) on insulin resistance in obese subjects with the metabolic syndrome [8]. We are currently testing the effects of lean-donor faecal microbiota transplantation on intestinal cholesterol fluxes. Using this human set-up, we hope to identify specific strains of intestinal bacteria associated with beneficial systemic effects on cardiometabolism, which could subsequently lead to the development of novel, microorganism-based intervention strategies.

In conclusion, an accumulating body of evidence relates imbalances in the composition of the intestinal microbiota to obesity and associated cardiometabolic disorders. The exact mechanisms by which the microbiota alters cardiometabolic homeostasis are being investigated in numerous research programmes. In the midst of this active area of research, there is an increasing need to closely apply Koch's' postulates to dissect causality from association. Thus, experimental findings on correlations between obesity and microbial communities should be corroborated by therapeutic studies in humans for their potential to prevent human disease and promote health.

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Transparency Declaration

The authors declare that there is no conflict of interest associated with this article.

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